

From Department of Molecular Medicine and Surgery
Karolinska Institutet, Stockholm, Sweden

BONE DISEASE AND DIABETES MELLITUS

Anna Ugarph-Morawski



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BONE DISEASE AND DIABETES MELLITUS

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By

Anna Ugarph-Morawski

Principal Supervisor:

Professor Kerstin Brismar
Karolinska Institutet
Department of Molecular Medicine and Surgery

Co-supervisor(s):

PhD, MD Maria Sääf
Karolinska Institutet
Department of Molecular Medicine and Surgery

Professor Per Wendell
Karolinska Institutet
Department of Neurobiology, Care Science and Society
Division of Family Medicine and Primary Care

PhD, MD Lars Kärvestedt
Karolinska Institutet
Department of Molecular Medicine and Surgery

PhD, MD Kristin Hjörleifsdottir Steiner
Karolinska Institutet
Department of Neurobiology, Care Science and Society
Division of Family Medicine and Primary Care

Opponent:

Professor Bente Langdahl
Aarhus University Hospital
Department of Endocrinology and Internal Medicine THG

Examination Board:

Associate Professor Anna Nilsson
Sahlgrenska University Hospital
Center for Bone Research

Professor Lisa Juntti-Berggren
Karolinska Institutet
Department of Department of Molecular Medicine and Surgery

Associate Professor Per Kristiansson
Uppsala University
Department of Public Health and Caring Sciences

TO MY CHILDREN DAVID, ALFRED AND ESTHER

ABSTRACT

Diabetes Mellitus (DM) and Osteoporosis (OP) frequently co-exist with advanced age and imply large health challenges worldwide. The last decades there has been a growing interest regarding fracture risk in DM. Currently used screening methods (Dual Energy X-ray Absorptiometry (DXA) and FRAX) underestimate fracture risk in diabetes patients. New methods for risk assessment are needed. In my thesis, we have studied the significance of neuropathy, the IGF-system and metabolic control in relation to bone mineral density and fracture risk in DM, both in a rat-model and in humans.

Study I: In an epidemiological register study of 24 605 patients, 12 551 men and 12054 women with T1DM the cumulative incidence of hip fractures was analyzed. Conclusion: Both men and women with T1DM have a several folds increased risk for hip fracture with higher risk in those with peripheral neuropathy.

Study II: Diabetic osteopathy and the IGF-system were analyzed in an animal model of mild T2DM, the Goto-Kakizaki rat, to assess the systemic as well as local bone and joint status. Conclusion: Bone mineral density (BMD) was lower in peripheral bone in diabetic compared to control rats and there were both systemic and local disturbances of the IGF-system

Study III: Bone and joint neuropathy were studied in the same diabetic rats and compared to controls to explore and define abnormalities of the peripheral nervous system in diabetic osteopathy according to nerve conduction velocity and neuropeptide expression in bone and joints. Conclusion: Rats with mild T2DM and neuropathy exhibited neuropeptidergic changes in the periphery, especially autonomic nerve deficits, which we suggest is an important factor underlying the development of regional osteopenia.

Study IV: In a prospective clinical study 66 subjects with T1DM or T2DM with peripheral poly-neuropathy (PNP) were followed for a mean of 11 years in T1DM and 8 years in T2DM to investigate fracture incidence and risk factors. Quantitative ultrasound (QUS) of calcaneus but not DXA spine or femur neck predicted future fractures. Fracture incidence was high in this cohort of poorly controlled diabetes subjects with the mean age of 58 years at inclusion. DXA spine T score was normal in both T1DM and T2DM, while T score DXA femoral neck and QUS of calcaneus were low, which correlated well. QUS of calcaneus and low levels IGFBP-5 predicted future fracture of the hip and foot. There was an inverse correlation between the incidence of any fracture and baseline levels of serum IGF-I. There were no gender differences or difference between type of diabetes in the incidence of fractures or risk factors.

In summary, the results of our studies show that peripheral local osteopenia occurs in diabetes associated with impaired IGF availability/activity and PNP independent of type of diabetes or gender and leads to an increased incidence of peripheral fractures. The pathogenesis behind fractures in DM is multifactorial. Neuropathy, microangiopathy and metabolic factors including the IGF system influence the development of peripheral osteopenia and fracture risk. This indicates that assessment of fracture risk should be follow in diabetes patients besides microangiopathy and neuropathy. QUS of calcaneus, serum levels of IGF-I and IGFBP-5 could be complementary screening methods for fracture risk assessment in subjects with diabetes and PNP.

LIST OF SCIENTIFIC PAPERS

- I. Miao J, Brismar K, Nyrén O, Ugarph-Morawski A, Ye W: Elevated hip fracture risk in type 1 diabetic patients: a population based cohort study in Sweden. *Diabetes Care*. 2005 Dec;28(12):2850-2855.
- II. Ahmad T, Ugarph-Morawski A, Lewitt MS, Li J, Sääf M, Brismar K. Diabetic osteopathy and the IGF system in the Goto-Kakizaki rat. *Growth Horm IGF Res*. 2008 Oct;18(5):404-411.
- III. Ahamad T, Ugarph-Morawski A, Li J, Bileviciute-Ljungar I, Finn A, Östenson CG, Kreicbergs A. Bone and joint neuropathy in rats with type-2 diabetes. *Regul Pept*. 2004 Jun 15;119(1-2):61-67.
- IV. Ugarph-Morawski, Sääf M, Brismar K. Predictors of fractures and bone disease in subjects with Diabetes Mellitus and Peripheral Neuropathy. Manuscript.

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LIST OF ABBREVIATIONS

BMD	Bone Mineral Density
BMC	Bone Mineral Content
BMI	Body Mass Index
BP	Blood Pressure
BUA	Broad Band Attenuation
CGRP	Calcitonin Gene Related Peptide
CI	Confidence Interval
DM	Diabetes Mellitus
DXA	Dual Energy X-Ray Absorptiometry
HbA1c	Glycated hemoglobin
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
i.p	Intraperitoneal injection
NCV	Nerve Conduction Velocity
NPY	Neuropeptide Y
PNP	Peripheral Neuropathy
QUS	Quantitative Ultra Sound
RIA	Radioimmunoassay
SHR	Standardized Hospitalization Ratios
SOS	Speed of Sound
SP	Substance P
WHO	World Health Organisation

1 INTRODUCTION

The incidence of Diabetes Mellitus (DM) is increasing worldwide, most significantly type 2 DM (T2DM) but also type 1 DM (T1DM). ⁽¹⁻²⁾ In 2013, 382 million people had DM. By 2035 this number is expected to rise to 592 million people. ⁽³⁾ T1DM affects 5-10% and T2DM 80-85% of the DM population. T1DM is characterized by absolute insulin deficiency due to beta cell dysfunction and has an autoimmune etiology. T2DM is characterized by deficiency in insulin secretion and insulin resistance. The dramatic increase in the prevalence of T2DM substantially depends on changes in lifestyle due to urbanization. Diabetes Mellitus is associated with several complications and comorbidities. The increased incidence of DM will impose a substantial burden on the health care system. To encounter this growing epidemic there is a need to appropriately allocate resources, identify individuals at risk of developing DM as well as individuals with manifest DM, to implement preventive measures of late complications. Until recently bone disease has not been included in the list of DM complications.

Osteoporosis (OP) is another public disease affecting millions of people worldwide. OP is defined as “a disease that is characterized by low bone mass, microarchitectural deterioration of bone tissue leading to enhanced bone fragility, and consequent increase in fracture risk”. ⁽⁴⁾ OP is a silent disease until a fracture occurs, why primary prevention is problematic. OP often remains underdiagnosed and undertreated. Accordingly, secondary prevention is the most common approach for fracture prevention.

The presence of OP related to DM has been less acknowledged and it's clinical relevance less obvious than that of other DM complications. However, there is a growing awareness that T1DM and T2DM both predispose to bone fracture. ⁽⁵⁾ The lifetime risk of an osteoporotic fracture in the general population in Rochester, Minnesota, has been estimated to almost 40% in white women and 13% in men from the age of 50 years onward. ⁽⁶⁾ However, a Swedish study reported substantially higher fracture incidence in an age- and sex standardized material. ⁽⁷⁾ A higher incidence is found in northern compared to southern Europe with differences in the epidemiologic pattern of hip fracture. ⁽⁸⁾ In subjects with T1DM there is an about six-fold increase in hip fracture risk and an about twofold increase in vertebral fracture risk compared to non-diabetic individuals. ⁽⁹⁾ T2DM is associated with an about two- to threefold increase in hip fracture risk compared with non-diabetic individuals, despite normal or high BMD. ⁽¹⁰⁾ In the ageing population, the likelihood to develop DM and/or OP are both increased.

According to current guidelines Dual Energy X-ray Absorptiometry (DXA) and Fracture Risk Assessment Tool (FRAX) are used to give information on the likelihood of future fractures. Previous reports demonstrate that those with T1DM generally have decreased Bone Mineral Density (BMD) measured by DXA and subsequent increased risk for osteopenia and osteoporosis, notably in the feet and femoral neck, being a risk for future fractures and Charcot Neuroarthropathy (CN) resulting in additional morbidity and mortality. ⁽¹¹⁾ Lately several reports have found that T2DM as well implies an independent risk factor for fracture. ⁽¹²⁾ Compared to control subjects, decreased BMD has been observed in T1DM, while contradictory results with higher, lower or normal values for BMD have been reported in T2DM patients. ⁽¹³⁻¹⁶⁾ In the non-diabetic population high BMD is considered a protective factor against fracture. However, in T2DM the association between BMD and fracture risk seems different, since T2DM patients have been reported to have higher risk of fracture despite normal or high BMD. ⁽¹³⁾ Previous studies have shown that the World Health Organization's (WHO) FRAX calculator underestimates fracture risk in individuals with T2DM. ⁽¹⁷⁾ Consequently, it is problematic to assess bone fragility in T2DM subjects by conventional methods. Bone strength includes several features: bone mineral density, bone structure, geometry, cortical architecture, trabecular architecture and bone turnover. DXA provides an estimation of bone density but does not capture other bone quality factors. Besides BMD, complications of DM (peripheral neuropathy (PNP), nephropathy, retinopathy), propensity to fall, hypoglycemia and decreased bone material properties influence fracture risk. ⁽¹⁸⁻²⁰⁾

The underlying mechanisms for increased fracture risk in patients with DM are indeed complex, multifactorial and are not fully established. Furthermore, T1DM and T2DM are heterogeneous diseases whose pathophysiological background and bone phenotype differ considerably. A selection of factors affecting bone metabolic status in DM will follow.

Metabolic control

There is evidence that glycemic control is associated with bone health. Poor glycemic control seems to be associated with increased fracture risk in both T1DM and T2DM subjects. ⁽²¹⁾ Chronic hyperglycemia has effects on both cellular and extracellular bone matrix, leading to impairment of osteoblast function. ⁽²²⁻²³⁾ In addition, high p-glucose generates the formation of advanced glycation end products (AGE). AGEs may play a potential role in the development of skeletal abnormalities by deteriorating bone quality and material properties, increasing stiffness and brittleness in bone. ⁽²⁴⁾ Bone consists of a collagen network (90%) that is susceptible to a variety of molecular alterations. Because of hyperglycemia, normal enzymatic crosslinking in collagen is decreased and AGE crosslinking increased, leading to more brittle bones that are less able to deform before fracturing. ⁽²⁵⁾ AGEs modify the organic bone material but not the mineral content in bone and can thus not be assessed by DXA. Accordingly, longer duration of the disease and worsened glucose control are associated with higher risk of fractures.

Insulin-like growth factor system

Insulin-like growth factor-I

Bone remodeling is regulated by systemic hormones and locally produced factors interacting to maintain bone mass. ⁽²⁶⁾ IGF-I is regarded as a key regulator of bone metabolism, both in skeletal growth and remodeling, in response to growth hormone (GH) exposure. GH may act directly on bone cells but most of its effects are mediated by IGF-I in both cortical and trabecular bone. In the adult skeleton, bone resorption and formation are coupled to maintain bone mass. Bone remodeling in the adult skeleton is necessary to remove potentially damaged bone. GH from the pituitary, parathyroid hormone (PTH) and insulin stimulates the production of IGF-I. ⁽²⁷⁾ IGF-I is a hormone with similar molecular structure to insulin and has important functions in regulating cell growth, differentiation and metabolism. It has glucose lowering and insulin sensitizing effects which is beneficial for glucose homeostasis. It exerts its actions by binding to the insulin-like growth-factor-I-receptor (IGFIR) and the insulin receptor on the cell surface. These receptors are present in most tissues, including bone. IGF-I is primarily produced by the liver acting as an endocrine hormone, but is also produced by other tissues acting in a paracrine/autocrine manner. ⁽¹⁰⁰⁾ In bone tissue IGF-I is synthesized in osteoblasts promoting proliferation and differentiation of osteoblasts, chondrocytes and collagen synthesis. The production of IGF-I is decreased in conditions of insulin deficiency and malnutrition. Diseases affecting the IGF system are associated with significant alterations in bone metabolism leading to decreased bone mass. ⁽²⁸⁾ IGF-I is biologically active only in unbound form and its action is modulated by a family of six binding proteins (IGFBP) with different functions. IGFBPs bind to IGF-I with an affinity equal to or greater than that of the IGF-I-receptor. When present in excess, tissue IGFBPs inhibit IGF actions by forming biologically inactive complexes that cannot bind to the IGF-I-receptors. The precise function of each IGFBP is still insufficiently understood, but they function as a storage pool of IGF-I, inhibit the action of IGF-I or potentiate the action of IGF-I. In this thesis, the focus will be on IGFBP-1, -4 and -5.

Insulin-like growth-factor binding protein-1 (IGFBP-1)

Phosphorylated IGFBP-1, produced mainly in the liver, inhibits IGF-I anabolic effects and influences the metabolic and mitogenic effects of IGF-I. ^(29, 101) Insulin inhibits the hepatic synthesis of IGFBP-1. ⁽³⁰⁾ High levels of IGFBP-1 has been associated to low BMD. ⁽³¹⁾ Conversely, IGFBP-1 is elevated in insulin deficiency, catabolic states and kidney failure.

Insulin-like growth-factor binding protein-3 (IGFBP-3)

IGFBP-3 is the major carrier of IGF-I in the circulation and is mostly regulated by GH and/or IGF-II. There are reports suggesting that patients with vertebral fractures have significantly lower levels of IGF-I and IGFBP-3 than controls without fractures. ⁽³²⁾

Insulin-like growth factor binding protein-4 (IGFBP-4)

IGFBP-4 is produced by the liver and in other tissues. In bone, it is produced by osteoblasts and is generally believed to inhibit the actions of IGF-I in bone cells by binding to the IGF-I receptor. ⁽³³⁾

Insulin-like growth factor binding protein-5 (IGFBP-5)

IGFBP-5 is the most abundant IGFBP in bone tissue secreted by osteoblasts and potentiates IGF-1 stimulated DNA- and protein synthesis in osteoblasts, chondrocytes, fibroblasts and smooth muscle cells. IGFBP-5 may also function as a growth factor by stimulating osteoblasts via an IGF-I independent mechanism. ⁽³⁴⁻³⁶⁾

Neuropathy

Diabetic peripheral polyneuropathy (PNP) is the most frequent neuropathy in western countries. Peripheral nerve function deteriorates with age in the general population, but more rapidly in patients with diabetes. ⁽³⁷⁾ PNP affects both sensory and autonomic neurons. Previous reports indicate that the autonomic nervous system is more susceptible to hyperglycemia and is therefore affected first in DM patients. ⁽³⁷⁾ The condition is related to duration of diabetes, metabolic control and occurrence of microvascular complications. There are several reports that indicate an important role of sensory and sympathetic nerve fibers in regulating bone remodeling. ⁽³⁸⁾ Neuropeptides from sensory and sympathetic nerve fibers in the periphery are involved in the regulation of bone metabolism. ⁽³⁹⁾

The autonomic neuropathy causes an increased blood flow, due to the development of arteriovenous shunts, contributing to increased bone resorption, osteopenia and bone fragility with subsequent fractures as consequence. Sensory neuropathy leads to loss of protective sensory capacity in bone and joints causing abnormal stress on the foot, imbalance in muscle, tendons and joints. Due to sensory deficit, repetitive trauma with micro-fractures and joint dislocations can initially pass undiscovered. These events contribute to the development of Charcot Neuroarthropathy (CN). CN also involves a local inflammatory process, gradual development of bone loss and permanent deformities of the foot skeleton. ⁽⁴⁰⁻⁴¹⁾ Additionally, sensory deficit causes instability and increased fall frequency with increased risk of any fracture.

In summary PNP, besides increased fall frequency, may affect bone through abnormal vasoregulation, but probably has direct effects on bone as well. Interactions between neuropeptides and growth factors as well as cytokines indirectly effect bone metabolism. The exact influence of the nervous system in bone maintenance is still unclear and further studies are required.

Neuropeptides

Substance P (SP)

SP is a neuropeptide acting as a neurotransmitter and as a neuromodulator. It is an important element in pain perception transmitting pain information from sensory nerves to the central nervous system. SP is released from sensory nerve fiber terminals and from inflammatory cells (macrophages, eosinophils, lymphocytes and dendritic cells). It is also found in the spinal cord (dorsal root ganglia) and in the brain. In addition, SP plays an important role in the inflammatory and angiogenetic phases of wound healing. Previous reports suggest that SP in the peripheral nervous system (PNS) contributes to maintain bone integrity, regulation of bone formation and resorption. ⁽⁴²⁾

Calcitonin Gene Related Peptide (CGRP)

CGRP is produced in nerves both in PNS and CNS, including skeletal nerve fibers in the periosteum and bone marrow. It is a potent vasodilator and is thought to control local blood flow. CGRP is derived mainly from sensory nerves, but also has autonomic functions. It mediates its effects on receptors found throughout the body including bone tissue. There are previous reports that CGRP is associated with bone formation. ⁽⁴³⁾ It also stimulates osteoblast proliferation, synthesis of growth factors (including IGF-1), cytokines, collagen and bone formation in vivo. ⁽⁴⁴⁻⁴⁶⁾

Neuropeptide Y (NPY)

NPY is one of the most abundant neurotransmitters in the brain and the autonomic nervous system of humans. It has vasoconstrictive and mitogenic effect on blood vessels and seems to be involved in blood pressure regulation and angiogenesis. Previous reports provide evidence that NPY fibers are present in bone, most commonly associated to blood vessels which indicate that NPY also has vaso-regulatory effects in bone. ⁽⁴⁷⁾ In the PNS, NPY appears to have pro-osteogenic effects. ⁽⁴⁸⁾ NPY is co-released with noradrenaline from sympathetic nerves. Sympathectomy has been shown to increase osteoclast number and modulate osteoblastic response to noradrenaline and PTH. ⁽⁴⁹⁻⁵¹⁾ Thus, presumably disorders in the PNS with subsequent altered expression of neuropeptides has adverse effects on bone metabolism.

2 HYPOTHESIS AND AIMS

Based on the hypothesis that Diabetes Mellitus type 1 (T1DM) and type 2 (T2DM) lead to bone tissue dysfunction including osteopathy and fracture due to metabolic disturbances and other DM complications, the present thesis included the following aims:

Study 1: To quantify the cumulative and relative risk of hip fracture in men and women with T1DM.

Study 2: To assess if abnormalities in the IGF-system in bone and joint may prove to underlie diabetic osteopathy, a study in an animal model of mild T2DM in rats was performed.

Study 3: To study if bone and joints are affected by neuropathy in T2DM, Nerve Conduction velocity (NCV) in the sciatic nerve and neuropeptides in these tissues were investigated in an animal model of mild T2DM in rats.

Study 4: To compare the methods Dual Energy X-Ray Absorptiometry (DXA) and Quantitative Ultrasound of Calcaneus (QUS) and investigate their ability to predict future fracture. In addition to study if bone parameters, measured by these methods, were associated with metabolic factors, including the IGF-system, in subjects with T1DM and T2DM and peripheral neuropathy (PNP).

3 MATERIAL AND METHODS

TABLE 1: SUMMARY OF STUDY DESIGN

Study I 24 605 DM1	Study II and III 18 GK rats + 21 Wistar female rats	Study IV 27 DM1 + 35 DM 2
<ul style="list-style-type: none"> •The Swedish Inpatient Register •Register of Total Population •Register of Domestic and Foreign Migration •The Causes of Death Register •Incidence •ICD 10 codes (cross-linkage within the Inpatient Register) •Fracture <ul style="list-style-type: none"> •hip (femoral neck, pertrochanteric) •Rethinopathy •Nephropathy •Neuropathy •Cardiovascular complications 	<ul style="list-style-type: none"> •Glucose tolerance test •Neuropathy <ul style="list-style-type: none"> •nerve conduction velocity •Bone mineral <ul style="list-style-type: none"> •DXA <ul style="list-style-type: none"> •humerus •vertebrae •tibia •Immunoassays <ul style="list-style-type: none"> •Insullin •IGF system serum •IGF-1 cortical bone and joint •Neuropeptides cortical bone, ankle, spinal cord, dorsal root ganglia •Immunohistochemistry <ul style="list-style-type: none"> •Neuropeptides cortical bone, ankle, spinal cord, dorsal root ganglia 	<ul style="list-style-type: none"> •Metabolic control <ul style="list-style-type: none"> •HbA1c •Neuropathy <ul style="list-style-type: none"> •Monofilament •Biothesiometry •Bone Mineral <ul style="list-style-type: none"> •Dual Energy X-ray Absorptiometry •Quantitative Ultrasound Calcaneus •Immunoassays <ul style="list-style-type: none"> •IGF-1 •IGFBP-1, -4 and -5 •Incidence <ul style="list-style-type: none"> •Medical records (ICD 10 codes) <ul style="list-style-type: none"> •Fracture •Charcot •Amputation •The Causes of Death Register •Mortality

3.1 STUDY I

3.1.1 ETHICS

This study was approved by the regional ethics committee at the Karolinska Hospital.

3.1.2 REGISTERS

Initially 25 221 records with a discharge diagnosis of T1DM were identified in the Swedish Inpatient Register. These records were further linked to the Register of Total Population, the Register of Domestic and Foreign Migration, and the Death Register. 171 records, with national registration numbers that could not be found in any of the registers, were excluded. In addition, 376 patients with date of emigration before the index hospitalization, 43 patients who died on the index hospitalization and 26 patients who already had hip fractures before

the index hospitalization were excluded. The final T1DM cohort included 24 605 subjects. (Table 1)

With minor exceptions, in-hospital medical services in Sweden have been exclusively public, organized by the community. In-hospital care registrations is in practice population based and referred to the county where the patients live, since patients have been constrained to use the hospitals in their county of residence. The Swedish In-patient Register was established by the National Board of Health and Welfare in 1964-1965, but most counties joined the registration later. The Register became nationwide 1987. Each record in the register corresponds to one hospital admission. In addition to the patient's national registration number (a unique identifier assigned to all Swedish residents), each record contains the dates of admission and discharge, codes for all surgical procedures and discharge diagnoses. During 1984-1986 five counties lacked national registration numbers for 1-2 years.

3.1.3 THE TYPE 1 DIABETES MELLITUS COHORT

The ICD coding before the 10th revision introduced 1997 did not allow us to separate T1DM from T2DM. Even after 1997 some patients coded as having T1DM had advanced T2DM that had developed insulin dependency. Thus, the age < 30 years at the first hospital admission for DM was used as an obligatory criterion. In an analysis, exclusively with patients hospitalized in 1998, when the ICD-10 codes were used in Sweden allowing differentiation between T1DM, T2DM and other DM, of 3 730 DM patients diagnosed at age 31 or younger, 3542 had a diagnosis of insulin-dependent type of DM. Accordingly, the age algorithm used in the study had a positive predictive value for T1DM of 95%. The obtaining of cohort data began at different dates in different counties depending on when full registration coverage without interruption had been established. The first county included was 1 January 1975 and the last 1 January 1989.

3.1.4 FOLLOW UP

The subjects in the cohort were followed from the index hospitalization for T1DM until occurrence of a first hospitalization for hip fracture, emigration, migration to a county without or with incomplete Inpatient Register coverage, death or the end of follow up (31 December 1998), whatever occurred first. The first hospitalization for hip fracture, if any, was identified through cross-linkage within the Inpatient Register. Complications of diabetes were identified in a similar way. Vital status and coverage by the Inpatient Register was ascertained by linkage to the registers of Death and Domestic and Foreign Migration.

3.1.5 STATISTICAL ANALYSES

Lifetime cumulative probability of developing hip fracture before the age of 65 years was calculated by using the Kaplan-Meier method. Standardized Hospitalization Ratios (SHR; the ratio of the observed to the expected numbers of first hospitalizations for hip fractures) served as a measure of relative risk. The entire Inpatient Register was used to calculate the background hospitalization rates for the diagnoses of interest (femoral neck, per trochanteric and multiple site fractures) and then for any hip fracture regardless of type in the general population by age (5 years sub-groups), sex and calendar period (every 2 years from 1975 to 1998). Stratum specific hospitalization rates were calculated by dividing the number of hip fractures by the corresponding number of general population at risk. The expected number of

hip fracture hospitalizations in the cohort was derived by multiplying the observed number of person-years in age, sex and calendar period strata by the corresponding stratum specific hospitalization rates. 95% confidence interval (CI) were calculated by assuming that the number of observed events followed a Poisson distribution.⁽⁵²⁾ We did not analyze multiple site hip fractures since no such case was found in our cohort.

Additional analyses were stratified according to follow up duration. χ^2 -test for linear trend was used to evaluate the time-risk relationship.⁽⁵³⁾ Presence/absence of DM complications was stratified. To the complication-negative strata, person-time experienced before the onset of complications was assigned.

Multivariate regression analysis (Poisson regression method) was used to analyze the independent effects of explanatory variables.

3.2 STUDY II AND III: ANIMAL MODEL, ANESTHESIA AND EUTHANASIA

3.2.1 ETHICS

The animals were cared for according to the Karolinska Institute's protocol and had ad libitum access to rat chow and tap water. All animal experiments were performed with approval from the local Animal Research Ethics Committee. X-ray and DXA examinations were made under Hyponorm anesthesia. The animals were euthanized by decapitation under anesthesia with Hyponorm (Janssen Pharma, Beerse, Belgium, 0.5 ml/kg, i.p) and samples were collected and frozen for immunoassay (IGF- and neuropeptide analysis) and by exsanguination for immunohistochemistry (morphological analysis of nerve fiber distribution).

3.2.2 ANIMAL MODEL

18 untreated female Goto-Kakizaki rats (GK rats) aged 12 months and 21 age-matched control Wistar rats (B&K Universal, Stockholm Sweden) were used. The GK rat has previously been developed in Japan by Goto et al. through selective inbreeding of Wistar rats based on impaired glucose tolerance,⁽⁵⁴⁾ thus the latter are eminent non-diabetic controls. The GK rats exhibit mild to moderate hyperglycemia of spontaneous onset as soon as 1 week after birth, associated with normal or slightly elevated plasma insulin levels, in the fed state, and an impaired insulin response to glucose. With advancing age, they develop insulin deficiency and chronic diabetes complications (neuropathy, nephropathy and retinopathy) although the metabolic abnormality is not severe.⁽⁵⁵⁾ Furthermore, the conduction velocity of peripheral nerves progressively declines with age and the ultrastructural changes in nerve fibers are similar to those seen in human diabetic subjects with neuropathy.⁽⁵⁶⁾ Previous studies have reported the appropriateness of this animal model for the study of neuropathy and osteopathy.⁽⁵⁷⁾ An animal model was used since the intention of study I and II was to analyze IGF-I and neuronal mediators in bone and joint why a human model was not considered ethically justifiable.

3.2.3 GLUCOSE TOLERANCE TEST

After an overnight fast the animals were given an intraperitoneal injection of glucose solution (2g/kg body weight) and tail blood glucose levels were determined (fasting, 30 and 120 min post-injection) to assess the diabetic status of the GK-rats, using a blood glucose analyzer (Accutrend, Boehringer Mannheim, Mannheim, Germany).

3.2.4 RADIOGRAPHY

X-rays of humerus and tibia were done using a dental X-ray machine (Heliodont DS, Siemens AG, Bensheim, Germany) and occlusal size films (Ektaspeed Plus, Eastman Kodak, Rochester, NY, USA). X-rays were digitalized with a scanner (Scanjet II, Hewlett Packard, Singapore) and printed at 20X magnification. The outer diameter of the cortex and medullary canal were measured in the distal third of the diaphysis with a precision caliper. Outer (periosteal) diameter divided by the inner (endosteal) gave the cortical thickness index. Mean of measurements by two blinded observers was calculated. The correlation between the observers was $r=0.65$ and inter-observer variation was 9%.

3.2.5 DUAL ENERGY X-RAY ABSORPTIOMETRY

Whole body DXA was performed using a clinical DXA machine with small animal software (QDR 4500, Hologic, Bedford, MA, USA). The whole body and regional high-resolution scans for humerus and tibia (whole bone, metaphysis, diaphysis) were analyzed by defining windows of fixed length, i.e. tibia: metaphysis in the proximal 15% of the bone length and diaphysis in the distal 15%; humerus: proximal 20% and middle 15%. The inter-scan variation was 3%.

3.2.6 RADIOIMMUNOASSAYS

Radioimmunoassay's (RIA) were done to analyze IGF-1, IGFBP-1 and IGFBP-4 in serum⁽⁹⁹⁾. Immunoassays were also done for quantification of IGF-1 and neuropeptides in tissue extracts. The animals were decapitated, blood was collected, centrifuged and the serum was frozen. Both ankles were collected, including the distal tibia and hind-foot. Vertebral bodies of L2-L5 vertebrae and the diaphyseal portions of both tibiae and femora were collected. Cortical bone was cleared of periosteum and bone marrow. Samples were immediately weighed and stored at -70 C. IGF-1, IGFBPs and neuropeptides were extracted from tissues according to the methodology previously described for neuropeptide extraction.⁽⁵⁸⁻⁵⁹⁾ Serum IGF-1 concentration was determined by RIA according to the methodology previously described by Bang et al.⁽⁶⁰⁾ Serum IGFBP-1 concentrations were measured using plate immunoassay as previously described by Povia et al.^(29, 61) IGFBP-4 was measured by RIA at the Laboratory of Growth and Development, California Pacific Medical Center Research Institute, San Francisco, USA, according to the method of Chelius et al,⁽⁶²⁾ who has reported serum IGFBP-4 levels in control rats from the current project. Serum insulin was measured using a commercial rat insulin RIA kit (Linco Research, St Charles, MO, USA).

3.2.7 STATISTICAL ANALYSES

Mean and standard deviation were used to summarize the measurements. In normally distributed data, Student's T-test was used for comparison of means between control and diabetic group. In skewed variables, group comparisons were done using Mann-Whitney test, and correlations were performed using log-transformed data. For significance p -value < 0.05 was chosen.

3.3 STUDY IV

3.3.1 ETHICS

This study was approved by the local ethic committee at the Karolinska Institute, Stockholm, Sweden, and satisfied the criteria of the World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects. All participants provided informed consent.

3.3.2 THE TYPE 1 AND TYPE 2 DIABETES MELLITUS COHORT

62 individuals with T1DM (n=27, 14 women, 13 men) and T2DM (n=35, 14 women, 21 men) diagnosed with peripheral neuropathy (PNP) were included in the cohort during 1995-1998. DM was defined as T1DM or T2DM according to American Diabetes Association, Classification and Diagnosis of Diabetes. ⁽⁶³⁾ At inclusion diabetes duration in T1DM subjects were 28 ± 13.6 years and in T2DM 13 ± 9 years. In T2DM subjects, 29 (83%) were treated with insulin, 4 (11%) with tablets and 2 (6%) with diet regime. Obviously all T1DM subjects were treated with insulin. Participants were followed from the time of inclusion until the first incident fracture, death or end of study (December 2015) resulting in 610 person-years with a median follow up time of 11 years in T1DM subjects and 8 years in T2DM subjects (range 1-20 years). All participants were consecutively referred to a multidisciplinary foot care team at department of Endocrinology at the Karolinska Hospital in Stockholm, Sweden. Clinical assessment and interview was made at base line. Clinical assessment measures included weight, height, blood pressure and examination of the feet. Body mass index ($\text{BMI}=\text{kg}/\text{m}^2$) was calculated based on weight and height. Hypertension was defined as blood pressure $\geq 140/90$ mmHg at examination or antihypertensive treatment. 16 individuals (7 T1DM, 9 T2DM) had Charcot foot deformations at inclusion. The participants were asked about smoking habits (present and previous), physical activity (active: normal daily life activity, including walks; inactive: immobilized or less than 10 minutes physical activity per day), history of thyroid disease, gastro-intestinal disease and heredity for osteoporotic fractures. 21 of the participants were postmenopausal (7 T1DM, 14 T2DM).

3.3.3 PERIPHERAL CIRCULATION

Examination regarding peripheral circulation was performed (pedal pulses a dorsalis pedis and a tibialis posterior, palpable or by Doppler). Individuals with severe macroangiopathy without pedal pulses (a dorsalis pedis and a tibialis posterior) and an ankle/arm index < 0.7 were excluded.

3.3.4 PERIPHERAL NEUROPATHY

Peripheral sensory neuropathy was tested regarding vibration and touch. Sensibility to touch was detected by performing monofilament tactility test (10gram Touch Test 5.07 Novo Nordisk, Copenhagen, Denmark) ⁽⁶⁴⁾ at four points at each foot. Vibration perception threshold using Biothesiometry ⁽⁶⁵⁾ at the metatarsophalangeal joint dig I. Clinical signs of autonomic neuropathy were evaluated with presence of dry, warm, shiny skin, loss of perspiration and absence of hair on the foot. A standard protocol designed to assess risk of future foot problems was used. ⁽⁶⁶⁾ All participants presented peripheral neuropathy, a majority both sensory and autonomic.

3.3.5 NEPHROPATHY

Nephropathy was defined as incident micro- or macro-albuminuria (3.0-30 g albumin/mol creatinine and > 30 g albumin/mol creatinine respectively) measured at two separate occasions. Urinary albumin was analyzed at the hospital laboratory with an immunological method, Beckman Array (Beckman Coulter Inc, Fullerton, USA). S-Creatinine (ref for women > 100 and men > 110 mmol/L) was analyzed at the hospital laboratory with a Creatinine aminohydrolas method, VITROS instrument (Johnson & Johnson, New Jersey, USA).

3.3.6 QUANTITATIVE ULTRASOUND OF CALCANEUS

The participants underwent QUS (Lunar Achilles Bone Densitometer) assessment of calcaneus in both feet at baseline. Mean value of both feet were calculated. In a subgroup (n=46, 74%) another QUS assessment was made after approximately 3 years. Quantitative ultrasound (QUS) is a bone health assessment tool that does not involve exposure to radiation, is affordable and the devices are portable, which could increase accessibility.⁽⁶⁷⁻⁷²⁾ The velocity (speed of sound=SOS, m/s) and frequency-dependent attenuation (broad band attenuation=BUA, dB/MHz) of the ultrasound signal is measured. Stiffness index is automatically calculated from broadband ultrasound attenuation and the speed of sound ($\text{Stiffness} = 0.67 \times \text{BUA} + 0.28 \times \text{SOS} - 420$). SOS and BUA are influenced by the bone tissue, reflecting its density, architecture and elasticity, which may be useful in evaluation of fracture risk. The T-value is equal to the standard deviation from the mean value (Stiffness) in healthy young adults of the same gender and the Z-value is equal to the standard deviation from the mean value in age matched controls.

3.3.7 DUAL ENERGY X-RAY ABSORPTIOMETRY

Measurements with DXA (Hologic ZDR-4500A) BMD of the spine (lumbar vertebrae L1-L4) and femoral neck were made in a subgroup of 34 participants (13 type 1 and 21 type 2). Standard operating procedures were followed for participant positioning. Scans were analyzed using Hologic software according to standard procedures. Characteristics in the subgroup which went through DXA measurements did not differ from the rest of the cohort. BMD is presented as percentage of the mean value (%BMD). The T-value is equal to the standard deviation (SD) from the mean value in young, healthy white males and females and the Z-value is equal to the SD from the mean value in age matched controls.

3.3.8 RADIOIMMUNOASSAYS

The IGF-system was assessed in a subgroup (n=30, 48%). IGF-I was determined in serum by RIA after separation of IGFs from IGFBPs by acid ethanol extraction and cryoprecipitation. To minimize interference of remaining IGFBPs, des (1-3) IGF1 was used as radioligand.⁽⁶⁰⁾ The intra- and inter-assay CV values were 4% and 11% respectively. Serum levels of IGF-I are age dependent, decreasing with age, thus IGF-I values are expressed as SD scores compared to healthy adult subjects. IGFBP-1 in serum was determined by RIA using a polyclonal antibody and human IGFBP-1 as standard.⁽²⁹⁾

Serum IGFBP-4 was measured by an ELISA (DSL active IGFBP-4) assay according to the manufacturer's directions. For all measurements, inter assay variability was less than 9% and intra assay variability was less than 7 %.⁽³³⁾

IGFBP-5 was measured by radioimmunoassay for IGFBP-5 using recombinant human IGFBP-5 as the antigen, tracer, and standard. ⁽³⁴⁾

The analysis of IGFBP-4 and -5 were made by Dr S Mohan, Loma Linda University, California.

3.3.9 MEDICAL RECORDS AND REGISTERS

Medical records regarding retinal photography were available after review of experienced retina ophthalmologists at the St Erik Hospital, Stockholm, Sweden. Retinopathy was classified as absent or present, ranging from mild non-proliferative retinopathy to proliferative retinopathy based on retina photographs.

At follow up data were collected from the participant's medical records. Incident fractures, amputations and development of Charcot foot deformations were based on registered ICD diagnosis. Incident fractures were verified by x-ray. Data on cause of death were obtained from The Swedish Health and Welfare Statistical Databases.

3.3.10 STATISTICAL ANALYSES

Statistical analysis was made in IBM SPSS Statistics. Pearson's correlation test was used to measure linear dependence between variables. Independent sample T-test was used for comparison between groups. Logistic regression was used for categorical variables with 95% confidence intervals (CI). Friedman test, a non-parametric test, was used for testing the difference between several related samples. Significance level was set at $p < 0.05$. Data are expressed as the mean \pm SD for continuous variables or percentages for categorical variables.

4 RESULTS

4.1 STUDY I

4.1.1 INCIDENT HIP FRACTURE IN PATIENTS WITH TYPE 1 DIABETES MELLITUS

This study included 24 605 subjects with T1DM (12 551 men and 12 054 women). Mean and median age at inclusion was 20,7 years. The cohort was followed for an average of 9.9 years (242 428 person years). During follow 121 incident hip fractures were recorded (70 among men, 51 among women). Anatomic site of fracture was the femoral neck in 64 (53%) and the per trochanteric area in 57 (47%) of the subjects. The mean age at diagnosis of first hip fracture was 43.1 years for women and 41.3 years for men.

4.1.2 CUMULATIVE RISK OF HIP FRACTURE IN PATIENTS WITH TYPE 1 DIABETES MELLITUS

The cumulative risk of hip fracture among subjects with T1DM, stratified by sex, increased significantly after age 30 years. After age 40 years it increased more rapidly in men compared to in women.

There was a significantly increased risk for hip fractures in both women and men with type 1 DM (SHR=9.8, 95% CI 7.3-12.9 and SHR=7.6, 95% CI 5.9-9.6, respectively). Among women the excess risk increased with follow up duration ($p < 0.02$). The excess risks were evident in men across different durations of follow up but without a clear trend. The risk for hip fracture increased further when diabetic complications (neuropathy, nephropathy, retinopathy, cardiovascular) were present. There was no significant difference in incident hip fractures between women and men (univariate and multivariate analysis). Subjects with diabetic complications, particularly neuropathy and nephropathy, had generally higher relative risks for hip fracture. (Fig 1)

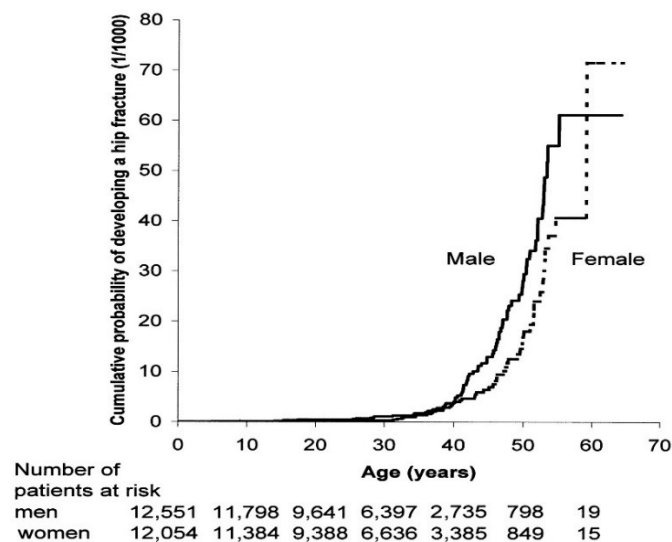


Figure 1: Cumulative probability (per 1,000) of developing hip fracture before the age of 65 years among type 1 diabetic patients, estimated by the Kaplan-Meier method.

4.2 STUDY II

4.2.1 DIABETES

To confirm the presence of diabetes in GK rats (female, 12 months old), intraperitoneal glucose tolerance test was performed. In control rats, the blood glucose levels peaked at 30 min and subsequently returned to baseline. In GK rats the blood glucose levels were significantly higher, throughout the test duration. (Fig 2)

4.2.2 RADIOGRAPHY

Both the periosteal and endosteal diameters of the long bone diaphysis of humerus and tibia were significantly increased in GK rats compared to Wistar rats. However, the cortical thickness of humerus and tibia was not altered, whereas the cortical thickness index (periosteal/endosteal diameter) was significantly decreased in GK rats compared to Wistar rats.

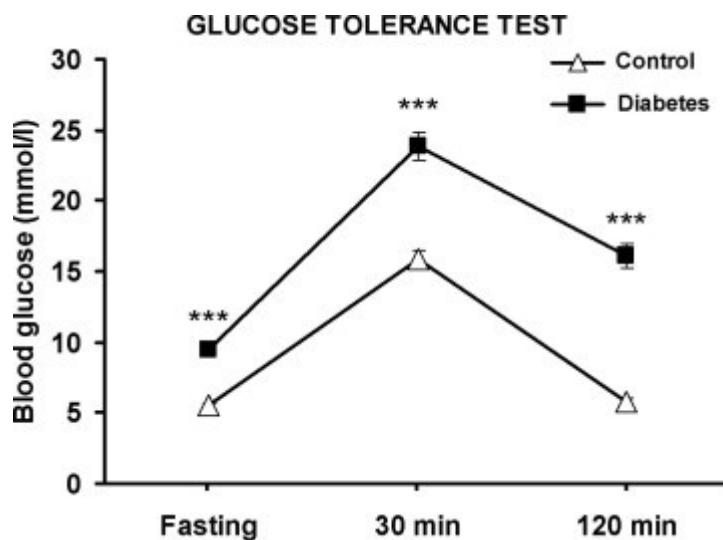


Figure 2: Blood glucose levels in the fasting state, 30 and 120 minutes after intra-peritoneal glucose injection ($p < 0.001$).

4.2.3 DUAL ENERGY X-RAY ABSORPTIOMETRY

In GK rats the whole-body bone area was reduced compared to Wistar rats, indicating a smaller skeleton with a lower Bone Mineral Content leading to unchanged Bone Mineral Density (BMD). BMD in GK rats was significantly lower in the metaphysis of tibia and humerus, compared to controls, in sub-region analysis. In the diaphysis BMD was increased in tibia and decreased in humerus. The weight in GK rats did not significantly affect bone parameters, according to ANCOVA.

4.2.4 THE IGF SYSTEM AND INSULIN

Serum

GK rats had significantly lower IGF-I levels in serum compared to Wistar rats, while IGFBP-1 and IGFBP-4 levels were significantly higher. (Table 2)

Bone and ankle

Local IGF-1 levels in diaphyseal cortical bone from tibia and femur was 38% lower in GK rats compared to Wistar rats ($p < 0.05$) but there was no difference in ankle and vertebrae. (Fig 3)

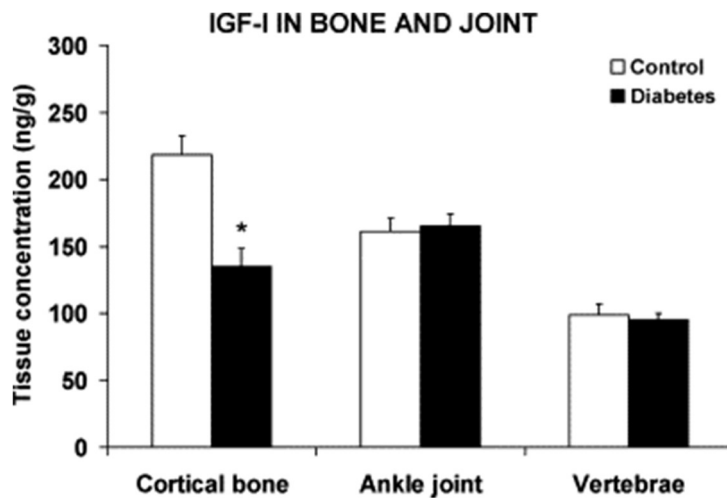


Figure 3: Mean IGF-I concentrations in cortical bone, ankle joint and vertebrae according to RIA ($p < 0.05$).

4.3 STUDY III

This study was performed in the same animals as in study II.

4.3.1 DIABETES

Higher blood glucose level in the fasting state, 30 and 60 min post-glucose tolerance test in GK rats compared to Wistar rats confirmed the presence of abnormal glucose tolerance in all GK rats. (Fig 2)

4.3.2 NEUROPATHY

Nerve function

Nerve Conduction Velocity (NCV) in the sciatic nerve was reduced by 16% in GK rats compared to Wistar rats.

Innervation

In both GK rats and Wistar rats immunohistochemical analysis showed the presence of sensory (Substance P; SP, Calcitonin Gene Related Peptide; CGRP) and autonomic (Neuropeptide Y; NPY) nerve fibers in bone and joints, however sparse. In bone marrow sensory nerve fibers were only occasionally seen and mostly peri-vascular. Autonomic nerve fibers were frequently observed in bone marrow, both related to blood vessels and as free nerve terminals. Autonomic neuropeptides (NPY) was also observed in large multinucleated megakaryocytes and occasional mononuclear hematopoietic cells of the bone marrow. In cortical bone, autonomic nerves were seen in vascular canals, most frequently near the endosteal surface. (Fig 4) The occurrence of sensory neuropeptides was also analyzed in dorsal root ganglia and the spinal cord. Immunoreactivity for both sensory neuropeptides (SP and CGRP) was found in lamina I and II of the dorsal horn and the cell bodies of the dorsal root ganglia. No clear difference could be demonstrated between GK rats and Wistar rats.

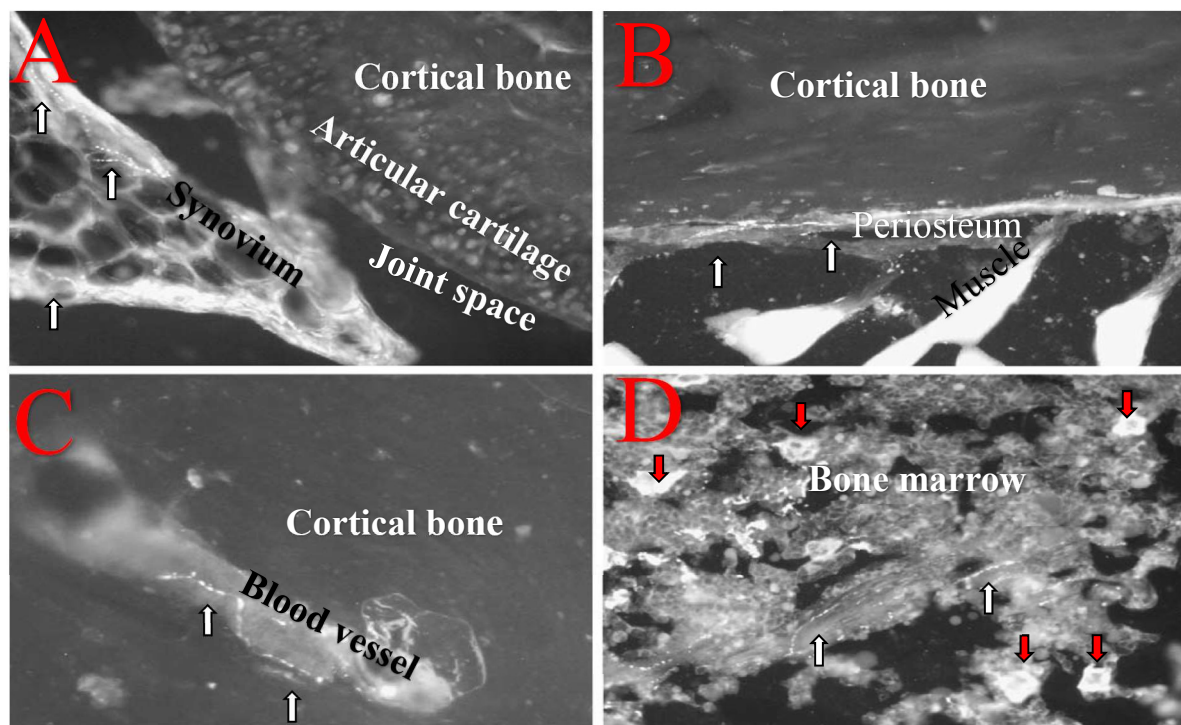


Figure 4: Innervation: Immunohistochemistry showed the presence of both sensory (SP and CGRP) and autonomic (NPY) nerve fibers in bone and joints. White arrows = nerve fibers. Red arrows = NPY positive cells (megakaryocytes, mononuclear hematopoietic cells)

A: Substance P, B: Calcitonin Gene Related Peptide, C and D: Neuropeptide Y

Neuropeptide levels

Radioimmunoassay (RIA) for sensory neuropeptides (SP and CGRP) in bone and joints did not demonstrate any significant difference between GK and Wistar rats. The levels of SP in periosteum and bone marrow were below detection limit and were excluded. In the spinal cord and dorsal root ganglia CGRP, but not SP, was significantly lower (-19% and -26% respectively) in GK rats. NPY concentrations in bone and joints were significantly decreased, notably, in cortical bone by -36%, in bone marrow by -66% and in ankle by -29%.

4.4 STUDY IV

4.4.1 STUDY SUBJECTS

62 (27 T1DM, 35 T2DM) subjects were included in the study. Table 2a+c summarizes the participant's characteristics at baseline and at follow up. Mean age at inclusion was 59 years (50 ± 13.6 years in T1DM, 65 ± 10.0 years in T2DM, $p < 0.0001$). There was no difference in mean age between women and men (data not shown). Mean DM duration was 20 years (28 ± 13.6 years T1DM, 13 ± 9.1 years T2DM, $p < 0.0001$) at inclusion. BMI was significantly lower in T1DM subjects compared to in T2DM subjects (24 ± 3 and 27 ± 6 respectively, $p < 0.007$). There were no significant differences in blood pressure, number of insulin treated subjects, HbA1c and serum creatinine levels between T1DM and T2DM subjects. All participants had PNP, poor metabolic control and approximately 50% had nephropathy. The majority had retinopathy and increased blood pressure.

Table 2a: Characteristics of the participants at baseline

 Characteristics of the participants at baseline. Mean \pm SD are shown for continuous variables

Characteristics	Type 1 DM (n=27) Mean (SD)	Type 2 DM (n=35) (Mean (SD)	p-value
<i>Baseline</i>			
Male (n, %)	n=13 (48)	n=21 (60)	
Age (years, (SD))	50 (13.6)	65(10.0)	0.0001
Age range (years)	29-74	40-78	
Diab duration (years, SD)	28 (14)	13 (9)	0.0001
Insulin treatment (n, %)	27 (100)	29 (83)	0.998
BMI (kg/m ² , SD)	24.15 (3.3)	27.49 (5.5)	0.007
SBP (mmHg)	145 (18)	146 (17)	0.944
HbA1c NGSP (%), SD)	8.7 \pm 1.6	8.3 \pm 1.4	0.296
HbA1c (mmol/mol, SD)	74 \pm 19	69 \pm 17	0.288
Creatinine (micromol/l)	89.4 (26)	85.8 (25)	0.594
Microalbuminuria (n, %)	16 (59)	19 (54)	0.991
Retinopathy (n, %)	25 (92)	25 (71)	0.051
Peripheral neuropathy (n, %)	27 (100)	35 (100)	1
Smoking (n, %)	12 (44)	20 (57)	0.323
Physically inactive (n, %)	6 (22)	13 (37)	0.210
Charcot at inclusion (n %)	7 (26)	8 (23)	0.780
IGF-1 (SD)	-1.99 (1.29)	-0.20 (1.96)	0.006
IGFBP-1 (microg/l)	104 (90.6)	39 (41.8)	0.012
IGFBP-4 (microg/l)	825 (259.7)	897 (91.0)	0.485
IGFBP-5 (microg/l)	635(86)	625 (91)	0.792

SD: standard deviation

BMI: Body Mass Index

SBP: Systolic Blood Pressure

DM: Diabetes Mellitus

IGF-1: Insulin-like Growth Factor 1

IGFBP: Insulin-like Growth Factor 1 Binding Protein

 Number of participants (%) are shown for categorical variables as YES/NO

Table 2b: Dual Energy X-ray Absorptiometry of the participants at baseline

Dual energy X-ray absorptiometry, Trabecular Bone Score, Quantitative ultrasound Calcaneus			
Bone variables	Type 1 DM (n=27) Mean (SD)	Type 2 DM (n=35)	p-value
QUS T-score (SD)	-1.77 (1.51)	-2.64 (1.36)	0.029
QUS Z-score (SD)	-0.64 (1.32)	-0.89 (1.30)	0.486
Stiffness	79.96 (16)	70.72 (15)	0.032
BUA (dB/MHz)	110.65 (11)	106.39 (10)	0.14
SOS (m/s)	1521.61 (33)	1499.71 (34)	0.022
DXA spine T-score	-0.67 (1.3)	0.30 (1.8)	0.089
DXA spine Z-score	-0.02 (1.2)	1.48 (1.6)	0.012
DXA spine BMD (g/cm ²)	1.00 (0.1)	1.14 (0.2)	0.067
DXA femoral neck T-score (SD)	-2.06 (0.88)	-1.82 (1.38)	0.567
DXA femoral neck Z-score (SD)	-1.06 (0.79)	0.002 (1.05)	0.006
DXA femoral neck BMD (g/cm ²)	0.718(0.08)	0.773 (0.19)	0.321
Total Tissue fat (%)	31±9	30±7	0.813
Lean body mass (%)	65±9	66±	0.660

QUS: Quantitative ultrasound of calcaneus. BUA: Broad Band Attenuation. SOS: Speed of sound

DXA: Dual Energy X-ray Absorptiometry. BMD: Bone Mineral Density

T-score: SD compared to young adults

Z-score: SD compared to age matched controls

Total tissue fat %: whole body composition

Table 2c: Characteristics of the participants at follow up

Characteristics of the participants at follow up. Mean \pm SD are shown for continuous variables

Characteristics	Type 1 DM (n=27) Mean (SD)	Type 2 DM (n=35) (Mean (SD)	p-value
Follow up time (years, SD)	11 (8)	10 (7)	0.278
Mean HbA1c over time (% , SD)	8.8 (1.5)	8.2 (1.2)	0.072
Any Fracture (n, %)	13 (48)	9 (26)	0.071
Hip fracture (n (%)	3 (11)	2 (6)	0.447
Foot fracture (n, %)	7 (26)	6 (17)	0.402
Mean age at any fracture (years, SD)	54 \pm 14	66 \pm 9	0.023
Diab dur. any fracture (years, SD)	39 \pm 12	16 \pm 10	0.0001
Total cases of Charcot (n, %)	10 (37)	14 (40)	0.812
New cases of Charcot (n, %)	3 (11)	6 (17)	
Amputation (n, %)	8 (30)	10 (28)	0.927
Death (n, %)	15 (60)	29 (83)	0,020
Mean age at time of death (years, SD)	70 \pm 10	75 \pm 9	0.077

SD: standard deviation

DM: Diabetes Mellitus

Number of participants (%) are shown for categorical variables as YES/NO

4.4.2 QUANTITATIVE ULTRASOUND AND DUAL ENERGY X-RAY ABSORPTIOMETRY (TABLE 2B)

In a subgroup of 34 participants DXA spine and femoral neck mean T-score was -0.67 ± 1.3 and -2.06 ± 0.88 SD respectively in T1DM subjects and 0.30 ± 1.8 and -1.82 ± 1.38 SD respectively in T2DM subjects. The difference between T1DM and T2DM subjects was only significant when corrected for age ($p < 0.012$ and $p < 0.006$ respectively, Z-score). The significant difference in T-score spine between women and men disappeared when corrected for age (Z-score). In femoral neck, there were no significant differences in neither T-score nor Z-score between women and men ($p=0.098$ and $p=0.520$ respectively).

Mean T-score of QUS was significantly lower in T2DM subjects compared to in T1DM subjects (-2.64 ± 1.4 and -1.77 ± 1.5 respectively, $p < 0.03$) but the difference disappeared when corrected for age (Z-score). When corrected for age there was no significant difference between women and men.

QUS T-score correlated significantly to T-score DXA spine and femoral neck ($p < 0.004$ and $p < 0.0001$ respectively). There was a significant correlation between QUS and DXA femoral neck even when corrected for age (Z-score, $p < 0.01$). There was a positive correlation between BMI and T-score DXA spine ($r=0.412$, $p < 0.015$) and femoral neck ($r=0.441$, $p < 0.009$), but no significant correlation was seen between BMI and T-score QUS calcaneus.

Thus, at baseline, age corrected DXA values (spine and femoral neck) was significantly lower in T1DM subjects but did not significantly differ between women and men. Age corrected QUS values did not differ significantly between T1DM and T2DM subjects or between women and men. There was a significant correlation between DXA (spine and femoral neck) and QUS (calcaneus) values.

4.4.3 FOLLOW UP EVENTS

Fractures

At follow up 22 of the participants (13 T1DM subjects, 9 T2DM subjects) had experienced one or multiple fractures (13 foot fractures, 3 lower limb fractures, 5 hip fractures, 2 vertebral fractures and 1 rib fracture). Fractures in the feet were most frequent. Median follow up time was 11 years in T1DM subjects and 8 years in T2DM subjects (range 1-20 years, 610 person years). Without adjustment for confounding factors there was no significant difference in fracture incidence between T1DM and T2DM subjects or between women and men. Mean age at the time of any fracture was 54 ± 14 years in T1DM subjects and 66 ± 9 years in T2DM subjects ($p < 0.023$) with no significant difference between women and men. T1DM subjects had significantly longer DM duration at the time of any fracture compared to T2DM subjects (39 ± 12 years and 16 ± 10 years respectively, $p < 0.0001$) with no significant difference between women and men. (Fig 2b. Table 1c) Neither smoking, HbA1c at inclusion, BMI, whole body tissue fat % nor gender was associated to increased incidence of any fracture. Logistic regression showed no increased incidence of any fracture in relation to measured T-score and Z-score QUS calcaneus, Stiffness, BUA, SOS, T-score or Z-score DXA spine and femoral neck at inclusion. However, when exclusively considering fractures of foot and hip, age adjusted QUS calcaneus (Z-score) was associated to fracture incidence ($p < 0.028$). Age adjusted DXA spine (Z-score) was higher in T2DM subjects who sustained any fracture than in those who did not. CN was present in 9 subjects with foot fracture.

Charcot Neuroarthropathy (CN)

At follow up there were 9 new cases of CN (3 T1DM subjects, 6 T2DM subjects) identified in the participants medical records (ICD 10 code). In total 24 of included participants had CN. There was no association between CN development and DXA, TBS or QUS when corrected for age. There was a positive association between CN development and T-score DXA spine, but the difference disappeared when adjusted for age (Z-score). In subjects with CN, 9 had a foot fracture, 21 had a foot ulcer and 11 were amputated.

Foot ulcer

In 41 of the participants foot ulcer was present during the study period (14 T1DM, 27 T2DM). In this group 10 subjects sustained a foot fracture, 21 subjects had CN and 17 were amputated. At follow up 36 (88%) of the subjects with foot ulcer had died.

Mortality

44 subjects had died at follow up. Mean age at time of death was 70 ± 10 years in T1DM subjects and 75 ± 9 years in T2DM subjects ($p=0.077$). There was no difference in mean age at time of death between women and men. Low QUS values in calcaneus were associated to increased mortality ($p < 0.001$), even when corrected for age ($p < 0.009$, Z-score). Of the deceased subjects 36 had a history of foot ulcer, 19 CN, 15 amputations and 8 foot fracture.

Cardiovascular disease was the most frequent cause of death. Causes of death are listed in Table 3.

Table 3: Causes of death

Cause of death	Number	% of death cases
Cerebrovascular event	11	25
Cardiovascular event	14	31.8
Malignancy	5	11.4
Diabetes complications	9	20.4
Septicemia	2	4.5
Traffic casualty	1	2.3
Intoxication	1	2.3
Collum fracture	1	2.3

Metabolic factors (BMI, HbA1c and the IGF-system)

At baseline, there was a positive association between BMI and T-score DXA spine ($r=0.412$, $p < 0.015$) as well as femoral neck ($r=0.441$, $p < 0.009$), but not to T-score QUS calcaneus. BMI was not associated to incident fracture at follow up. However, T1DM subjects with any fracture had significantly lower BMI than T2DM subjects with any fracture.

HbA1c at baseline did not correlate to DXA, QUS or TBS. At follow up there was a tendency to higher mean HbA1c over time in subjects with any fracture ($p=0.081$).

IGF-I SD was significantly lower and IGFBP-1 significantly higher in T1DM compared to in T2DM subjects ($p < 0.006$ and $p < 0.012$ respectively) at baseline. There was no difference in IGFBP-4 or IGFBP-5 levels in between T1DM subjects and T2DM subjects. (Table 2a). IGF-I SD was positively associated with DXA spine ($r=0.459$, $p < 0.042$) and femoral neck ($r=0.437$, $p < 0.05$) when adjusted for age, but not to QUS calcaneus. There was an inverse correlation between IGF-I SD and incidence of any fracture ($p < 0.035$), chiefly in T2DM patients. No association was seen between overall fracture incidence and IGFBP-4 or IGFBP-5. However, when exclusively considering fractures of foot and hip, there was a significant inverse association with IGFBP-5 ($p < 0.047$). (Fig 6). HbA1c at baseline did not correlate with IGF-1, IGFBP-1, IGFBP-4 or IGFBP-5.

Figure 5: IGF-I SD (age corrected levels) in T1DM and T2DM patients with and without any fracture.

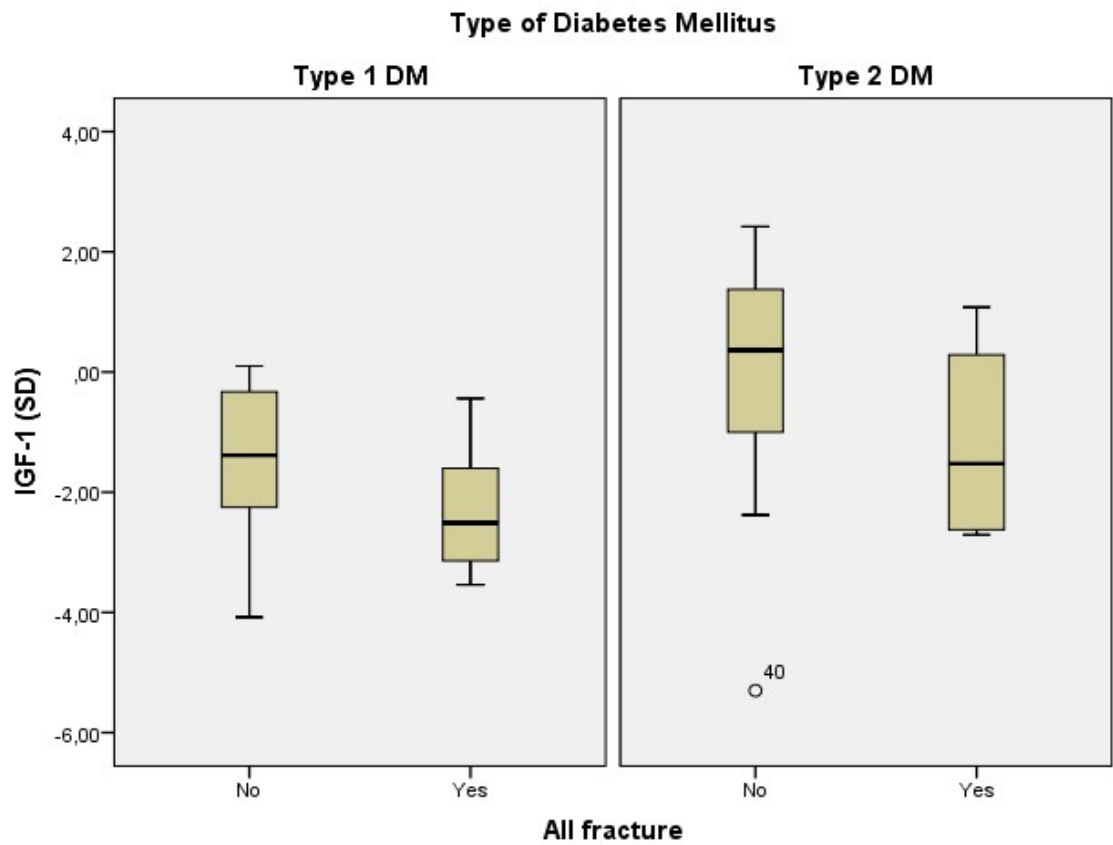
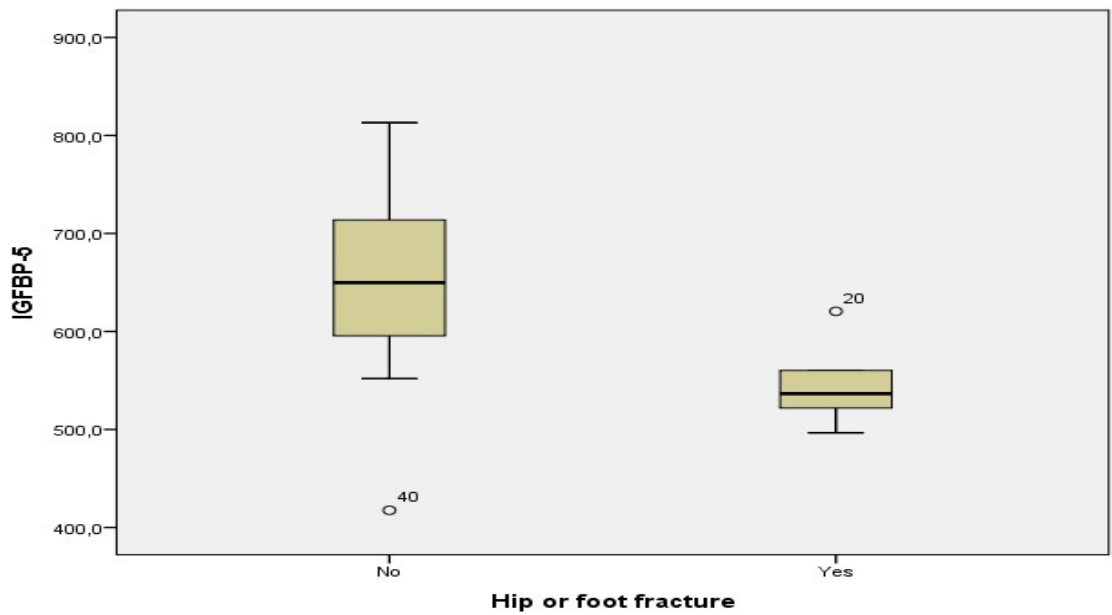


Figure 6: Serum levels of IGFBP-5 in subjects with and without hip or foot fracture ($p < 0.047$).



5 DISCUSSION

The results of this thesis are based on experimental animal studies, clinical studies and epidemiologic studies. The aim of our studies was to investigate the fracture incidence and possible predictors of fracture in patients with diabetes.

5.1 FRACTURE INCIDENCE

In the retrospective cohort study on T1DM patients we observed a distinctly increased hip fracture risk among men and women who had been hospitalized for T1DM as compared to the general population. Concurrent presence of diabetes complications such as neuropathy, nephropathy, retinopathy or cardiovascular disease indicated additional fracture risks ranging from 17- to 42-fold. This is in accordance with subsequent reports.⁽⁷³⁾ The fact that only patients with T1DM diagnosed before the age of 30 years were included in study I may have resulted in inclusion of subjects with a longer duration of diabetes and thus more complications than in previous reports.⁽⁷⁴⁾ The high incidence of hip fracture before the age of 65 in study I, elucidate the necessity for development of methods for primary prevention in patients with T1DM.

The association between fracture risk and neuropathy was further studied in the prospective, clinical study on T1DM and T2DM patients with PNP. We report a high incidence of any fracture, most prominent in the foot and hip, in this prospective clinical study. All participants had diabetes complications which presumably result in an overall higher fracture risk than in other studies.⁽⁷⁵⁾ The incidence of any fracture did not significantly differ between T1DM and T2DM patients, but tended to be higher in T1DM patients ($p=0.071$). The lack of a significant difference in fracture incidence between the T1DM and the T2DM patients in study IV could be due to a lack of statistical power. Regarding fractures of hip and foot there were no differences between T1DM and T2DM. Fracture incidence in study IV might be underestimated due to high mortality, high amputation frequency and undiagnosed fractures. In accordance with previous reports, the T1DM patients were younger, had longer DM duration and lower BMI than the T2DM patients in study IV.⁽⁷⁶⁾ Interestingly, in both study I and IV, the risk of fracture in men exceeded that in previous reports. In the general population fracture risk is higher in women compared to in men.⁽⁹⁷⁾ However, our results and available literature indicate that in the diabetes population men seems to be less protected from fractures.⁽⁹⁸⁾ Thus, the gender difference in fracture risk is different in diabetes patients compared to the general population. It is known that gender differences also differ regarding cardiovascular disease in diabetes patients as compared to the general population.

The prospective clinical study is associated with several limitations. The sample size was not large enough to draw definitive conclusions concerning fracture risk. The material consisted of Caucasians only. Moreover, the participants were all included from the Foot clinic at the Karolinska University Hospital which is a tertiary hospital for diabetes and OP. The severity of disease in these patients is probably more pronounced than in the general diabetic population. We have not accounted for the frequency of falls or hypoglycemic events. Since the start of our study new bone turnover markers and methods of measuring bone quality have been discovered and have therefore not been considered in this report. Advantages in study IV are the long follow-up time, the clear distinction between T1DM and T2DM, inclusion of both men and women and that no participant was lost to follow-up. The results indicate that PNP is a risk factor for bone disease and incident fractures in T1DM and T2DM

patients. It seems that these patients have regional bone disease since the incidence of fractures in the foot and hip were substantially higher than the risk for vertebral fractures. The vertebrae, metaphysis of long bones and calcaneus consists of trabecular bone.

5.2 METABOLIC FACTORS

5.2.1 Body mass index (BMI)

High BMI is considered protective of fracture in non-diabetic individuals. Our results show that T1DM subjects with any fracture had lower BMI compared to those without fractures (study IV). In both the animal model of T2DM and in T2DM patients, body weight or BMI was not associated to BMD or fracture incidence (study II, III and IV). Thus, our results indicate that in T2DM subjects with complications, e.g. PNP, BMI does not seem to be sufficiently protective against fractures.

5.2.2 HbA1c

We did not find any significant association between HbA1c and bone parameters or fracture incidence. However, our data indicate that tight metabolic control is of importance considering the covariation between hip fracture and other DM complications. Furthermore, T1DM patients born before 1950, who presumably had T1DM a considerable time before the importance of metabolic control was identified, had extensively higher fracture risk (Study I). Hyperglycemia give rise to oxidative stress, hypoxia and the formation of AGEs. The formation of AGEs increases in the general population with age, but hyperglycemia accelerate this process in diabetic patients. AGE formation occurs in proteins in all tissues, including bone. The microcirculatory disturbances and microangiopathy seen in diabetes supposedly are associated to long term metabolic control and not to current HbA1c value.

5.2.3 The IGF system

The results of study II and IV indicate that the IGF system is disturbed, both systemically and locally, in DM. The animal model of T2DM showed reduced IGF-I activity with low IGF-I levels in both serum and cortical bone, high levels of IGFBP-1 and IGFBP-4 in serum and affected response of trabecular bone to local IGF-I. Abnormalities in the IGF system presumably, and to a certain extent, cause the reduction in BMD of trabecular bone in the metaphysis and the vertebrae of T2DM rats. The IGF-signaling pathway is crucial for bone acquisition and bone remodeling.⁽⁷⁷⁾ IGF-I has osteogenic properties and stimulates osteoblast differentiation and proliferation thereby playing a key role in maintenance of bone mass. The results from the prospective clinical cohort study (study IV) show that low serum IGF-I levels were associated with incidence of any fracture, confirming our previous study on rats with T2DM (GK-rat) and PNP reporting an association between decreased levels of IGF-I (serum, bone) and regional osteopenia. This is consistent with previous reports of an association between decreased levels of IGF-I and fractures in patients with diabetes.⁽⁷⁸⁾ Normal ageing involves a decline in S-IGF-I. Our results showing low systemic and local IGF-I levels in T2DM rats with osteopathy and an association between low systemic IGF-I levels and fracture incidence in T1DM and T2DM patients, indicate that diabetes resemble premature ageing of bone tissue.

In the animal model of T2DM serum levels of IGFBP-1 and IGFBP-4 were elevated. These proteins are expected to impair IGF-I action on bone and have a direct effect on osteoblasts

inhibiting bone formation.^(33, 79) This indicates that the IGFBPs may affect bone metabolism. However, in the prospective clinical study, IGFBP-1 and -4 did not correlate with bone parameters or fracture incidence. Interestingly, low IGFBP-5 levels seemed to predict risk of fractures in the foot and hip. Our results agree with previous reports that IGFBP-5 potentiates IGF-I effects and functions as a growth factor by stimulating osteoblasts via an IGF-1 independent mechanism.⁽³⁴⁾

Reports indicate that IGF-1 also affects the synthesis of nitric oxide (NO), which is involved in bone metabolism.⁽⁸⁰⁾ NO regulates bone metabolism by decreasing resorption, increasing formation and reducing oxidative stress. An association between cardiovascular disease and osteoporosis has previously been suggested and endothelial dysfunction may be an underlying mechanism.⁽⁸¹⁾ The incidence of micro- and macrovascular disease was high in study IV suggesting a link between impaired bone metabolism, the IGF system and endothelial dysfunction. IGF-I decreases in patients with poorly controlled diabetes suggesting that optimal metabolic control may decrease the risk of future fractures.

5.2.4 Peripheral neuropathy

Our results consistently indicate an association between PNP and bone disease in diabetes. PNP is associated with impaired microcirculation, microangiopathy and subsequent hypoxia, contributing factors in diabetes bone disease. In the prospective population-based cohort study of T1DM patients (Study I) the presence of PNP indicated excess risk of hip fracture that ranged from 17- to 42-fold. In the animal model of T2DM with osteopathy (Study III), NCV was reduced and we could demonstrate neuropeptidergic changes in bone and joints. In the prospective clinical study of T1DM and T2DM (Study IV), all participants had PNP and the fracture incidence was substantially high. There was also a high incidence of CN. In the general diabetes population CN is present in about 1% of diabetics (McInnes AD et al). It is known that diabetes and PNP are the most common precursors of CN. The pathogenesis of CN involves neurovascular disturbance due to pathological innervation of the blood vessels and repetitive micro-trauma of the bone and joints in the foot. Dysregulation of blood flow in bone due to autonomic neuropathy causes increased peripheral blood flow and bone resorption, osteopenia and local bone destruction. CN is characterized by presence of PNP, inflammation, rapid bone resorption and osteopathy. Sympathetic denervation causes increased bone perfusion and bone resorption linked to microvascular abnormalities. Microvascular changes in diabetes are enhanced by inflammation and may be associated to increased risk of incident fracture and CN.⁽⁹²⁾ PNP may be one factor causing the regional osteopathy seen in our studies. Presumably, progressive denervation leading to destruction of bone tissue in the foot and ankle increases the risk of fractures in the extremities, e.g. in the foot and hip, but not vertebral fractures.

In rats with T2DM the neuropeptidergic changes pertained to the autonomic mediator NPY, whereas the sensory neuropeptides, SP and CGRP, were normal in the periphery. CGRP was decreased in dorsal root ganglia and spinal cord. This is in accordance with previous reports in which the onset of autonomic neuropathy precedes that of sensory dysfunction in patients with diabetes.⁽⁸²⁻⁸³⁾ The GK rat presents a mild T2DM why, hypothetically, the decreased CGRP in dorsal root ganglia and spinal cord in the present study represents an early sign of sensory deficit eventually propagating to bone and joint in a more severe stage of the disease.

NPY is widely distributed in the central (CNS) and peripheral nervous system (PNS) and there are reports that NPY, centrally and peripherally, attenuates stress-induced bone loss.⁽⁸⁴⁾ Chronic stress is associated not only with psychiatric disease but also somatic disease, such as diabetes. In the periphery NPY is co-released with noradrenaline from noradrenergic neurons. Our results suggest that NPY has an anti-resorptive or pro-osteogenic effect on bone, consistent with previous reports.^(45, 85-87) Given the suggested bone protective role of NPY during chronic stress, NPY deficiency in DM and PNP may be a contributing factor underlying the development of regional osteopenia.

We suggest that the presence of PNP in DM patients should be considered as a risk factor for future fracture.

5.2.5 Bone parameters

In the animal model of T2DM DXA measurements showed significantly lower BMD in the metaphysis of both tibia and humerus in DM rats compared to controls. The BMC and BMD according to DXA were significantly reduced chiefly due to bone loss in vertebrae and metaphysis of long bones. Measurements with peripheral quantitative computed tomography have previously shown that the decrease in volumetric BMD almost exclusively affected trabecular bone (vertebrae, metaphysis) in the GK rat.⁽⁸⁸⁾ The metaphysis consists primarily of trabecular bone. The regional osteopenia seen in study II was associated with decreased levels of IGF-I in serum and in bone. In the prospective clinical study, IGF-I was associated with increased fracture risk in both T1DM and T2DM patients. Our results indicate that bone disease and fracture risk, at least to some degree, are caused by abnormalities in the IGF system. According to previous reports it seems like trabecular bone is more affected than cortical bone.⁽⁸⁹⁾ Our results indicate that trabecular bones in the extremities are more affected than in the spine, which may be due to PNP and dysfunctional vaso-regulation in the periphery.

There was no significant difference in DXA T-scores at the femoral neck between T1DM and T2DM patients with PNP. DXA of spine and femoral neck did not predict future fracture in this cohort. Considering the small sample size this might be due to lack of statistical power.

In study IV we performed QUS of the calcaneus in all patients. The parameters measured by QUS were low in both T1DM and T2DM patients. The gender difference disappeared when corrected for age. Low QUS of the calcaneus was associated with increased incidence of fracture in the foot and hip. Presumably the parameters of QUS calcaneus can reflect the bone structure of proximal femur and there are previous reports showing that QUS of calcaneus can predict risk of hip fracture⁽⁹⁰⁾ and that PNP is related to lower hip BMD and calcaneal QUS values.⁽⁹¹⁾ Our results indicate that bone material properties, not captured by DXA, are altered in diabetes, particularly when PNP is present. Low QUS values, but not DXA, were associated to high fracture risk and mortality at follow up, in this population with multiple diabetes complications. Considering the hypothesis that AGEs in bone increase with age and long duration of diabetes, QUS may reflect the AGE content in bone and soft tissue of the foot, which is not captured by DXA.

6 CONCLUSIONS

Both female and male subjects with T1DM are at increased risk for hip fracture, more common in those with neuropathy. Subjects with T1DM and T2DM and PNP were at high risk for bone metabolic deterioration and consequent fractures, especially in the hip and foot. The co-occurrence with other diabetic complications suggests that tighter metabolic control might be of importance in the prevention of diabetic hip fracture. At follow up, there was also a tendency to association between HbA1c over time and incidence of any fracture.

Female 12 months old GK rats with mild T2DM and neuropathy exhibited neuropeptidergic changes in the periphery with more pronounced autonomic neuropathy than sensory neuropathy. This is in accordance with observations in diabetic subjects, reporting the onset of sub-clinical autonomic neuropathy prior to development of sensory dysfunction. The results suggest that local neuropeptidergic deficit in diabetes may prove to be an important factor underlying the development of regional osteopathy. These GK rats exhibited regional osteopathy and disturbances in the IGF system. IGF-1 is an anabolic factor for bone and low levels have been shown to cause osteopenia. Both low systemic and local bone levels of IGF-1 were related to local osteopenia. Accordingly, in the prospective clinical study of T1DM and T2DM low IGF-I SD serum levels were associated with the incidence of any fracture. When considering only fractures in foot and hip there was an inverse correlation between IGFBP-5 and fracture incidence.

DXA spine was normal while DXA femoral neck and QUS calcaneus were low in T1DM and T2DM independent of gender, suggesting regional impairment in bone turnover in diabetes. Our results indicate that QUS calcaneus and not DXA might be used to screen for osteopenia and osteoporosis in subjects with diabetes and peripheral neuropathy and predict fractures in foot and hip. Hypothetically, QUS might reflect AGE accumulation in bone and soft tissue, not captured by DXA.

In summary, currently used screening methods (DXA and FRAX) underestimate fracture risk in diabetes subjects. Our results indicate that the peripheral nervous system and the IGF system are involved in bone metabolism in diabetes and impairment in these systems can explain increased fracture risk. The risk of peripheral fractures seems to be increased more than axial fractures in diabetes patients with PNP. This conforms to proposed regional osteopathy. Tight metabolic control and treatment normalizing the IGF activity already early after diabetes diagnosis and over time may have positive effect on the peripheral nervous system, AGE formation and resistance of bone to fracture. QUS of calcaneus, serum levels of IGF-I and IGFBP-5 could be complementary screening methods for fracture risk assessment in subjects with diabetes and PNP.

7 FUTURE PERSPECTIVES

The incidence of DM, particularly T2DM, is increasing globally. Simultaneously, osteoporosis and increased fracture risk is frequent in many populations. In the ageing population these diseases generate a tremendous burden for the individual and the health care system. The results presented in this thesis indicate that both T1DM and T2DM are associated to increased fracture risk. The pathogenesis is multifactorial and complex. It is of importance to develop adequate screening methods to identify individuals at risk for future fracture and implement preventive measures. An increased understanding of the pathologic mechanisms leading to incident fracture is anticipated. A majority of T2DM patients are followed up in primary care. Evidence based screening tools and medical treatments are required to offer preventive and equal care for the broader population.

Immigrant from the Middle East have higher prevalence and incidence of T2DM compared with native Swedes.⁽⁹³⁾ There are reports that low serum 25-hydroxyvitamin D levels increase risk of T2DM and increase fracture risk.⁽⁹⁴⁻⁹⁵⁾ We have started a prospective cohort study on newly diagnosed T2DM subjects without DM complications. The participants are recruited from primary care units in the Stockholm area. In one group we include 100 patients with non-European origin and 100 controls matched for age, gender and origin. In the other group we include 100 native Swedish patients and 100 controls matched for age, gender and origin. The participants go through clinical examination (BMI, waist circumference, blood pressure, foot examination, eye examination) and a questionnaire regarding socioeconomic status, education, physical activity, diet, alcohol consumption, smoking habits, clothing (veiled) and heredity for T2DM and/or fracture. We will assess blood samples regarding metabolic control, vitamin D, vitamin D receptor, PTH, calcium, creatinine, GFR, adiponectin, sclerostin, osteoprotegrin, lipids and inflammation markers. The intention is to investigate AGE-levels, bone quality with microindentation method and assessment with QUS calcaneus. We will consider diabetic medication, frequency of hypoglycemia and risk of falls.

In summary future prospective studies are required to determine the optimal management strategies for fracture prevention in diabetes.

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